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Quantitative autoradiographic localization of NMDA, quisqualate and PCP receptors in the frog tectum

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An organizing role for the *N*-methyl-D-aspartate (NMDA) receptor/channel has been suggested in the development of the retinotectal projection in *Rana pipiens*. The regional distributions of NMDA, phencyclidine (PCP) and quisqualic acid (QA) receptors were quantified using in vitro autoradiography in the tectum of normal and surgically produced 3-eyed juvenile frogs. NMDA and QA receptor binding was highest in the pretectum. Of the tectal layers, the superficial retinotectal synaptic zone, layer 9, had the highest amount of NMDA and QA receptor binding. Moderate binding was observed in layer 5, with little binding in the cellular layer 6. No specific [³H]*N*-(1-[2-thienyl]cyclohexyl) piperidine ([³H]TCP) binding was observed in any of the tectal regions.

The development of the retinotectal projection in frogs involves a constant rearrangement of the retinotectal synaptic contacts in order to maintain a high-fidelity retinotopic map despite the mismatched growth patterns of the retina and optic tectum¹⁶. Studies using tetrodotoxin to block action potentials in the optic nerve demonstrate that the mechanisms for synaptic rearrangement or fine tuning require retinal ganglion cell (RGC) activity¹⁷. Recently, chronic treatment of the optic tectum with the *N*-methyl-D-aspartate (NMDA) receptor antagonist aminophosphonovaleric acid (APV) has been shown to cause desegregation of the ocular dominance columns in surgically produced 3-eyed tadpoles³ and disruption of the refined retinotopic map in 2-eyed tadpoles⁴. Furthermore, treatment of optic tecta of 3-eyed tadpoles with NMDA itself appears to increase stripe segregation, as is evidenced by stripes with sharper borders³. These data suggest that the NMDA receptor, a subclass of glutamate receptors,

is involved in the formation and maintenance of the topographic retinotectal projection in the frog.

Glutamate is a favored neurotransmitter in the RGCs of lower vertebrates. In goldfish, the broad scale glutamate receptor antagonists, kynurenate, γ -D-glutamylglycine and *cis*-2,3-piperidine dicarboxylate, block evoked field potentials in slices of optic tectum¹⁰. Similarly in the frog, evoked potentials recorded from the optic tectum following optic nerve stimulation are blocked reversibly by kynurenate⁶. Enucleation results in a decrease in high-affinity glutamate uptake in the optic tectum¹⁸. Based on these data and the work summarized above, we hypothesized that binding sites for glutamate or other excitatory amino acids are located in the retinotectal synaptic zone of the optic tectum. Indeed, a recent study has shown the presence of glutamatergic ligand binding sites in the optic tectum of the goldfish⁸ although glutamate binding sites have not yet been demonstrated in the frog optic tectum.

Glutamate receptors are typically divided into

NMDA and non-NMDA subtypes according to their ability to be activated selectively by the ligand NMDA and blocked selectively by the antagonist APV (see ref. 14 for review). Non-NMDA glutamate receptor subtypes can be further divided into quisqualate (QA)- and kainate-sensitive binding sites, based on the selective affinities of these ligands. NMDA receptor activation is blocked non-competitively by phencyclidine (PCP) receptor ligands including TCP and MK-801 (ref. 9) and NMDA and PCP receptors are colocalized in rats¹². Therefore it is thought that the two receptors are part of the same receptor/channel complex¹⁴. Similarly, NMDA and non-NMDA receptors often appear colocalized in electrophysiological and anatomical studies^{7,11}. We examined the regional distributions of NMDA- and quisqualate-sensitive [³H]glutamate binding sites and [³H]*N*-(1-[2-thienyl]cyclohexyl) piperidine ([³H]TCP) binding sites within the tectum of normal and 3-eyed juvenile frog brains using quantitative autoradiography.

Normal ($n = 4$) and surgically produced 3-eyed ($n = 3$) juvenile *Rana pipiens* frogs (1–3 months post-metamorphosis) were anesthetized by immersion in 0.1% methanesulfonic acid (MS222, Sigma). Brains were rapidly removed and frozen immediately on dry ice. Radiolabelling of each receptor class was carried out on alternate 20 μm coronal brain sections that were thaw-mounted onto gelatin-coated slides. NMDA- and QA-sensitive [³H]glutamate binding assays and [³H]TCP binding assay were performed as described in the legend of Fig. 1.

Tissue sections were apposed to tritium-sensitive film (LKB Ultrafilm) for 35 days. A set of radioactive standards (ARC Tritium) calibrated against brain pastes with known amounts of tritium was co-exposed with each film. Quantitative analysis of the resulting autoradiograms was performed densitometrically using a microcomputer based video densitometer system (Imaging Research, St. Catharines, Ont.). Optical density values were converted to pmol/mg protein using a computer-generated polynomial regression analysis which compared film densities produced by the tissue sections to those of radioactive standards¹⁵. Five or more autoradiographic sections were analyzed per animal and 10 readings were taken per area. Data represent specific binding (90% specific binding in [³H]glutamate

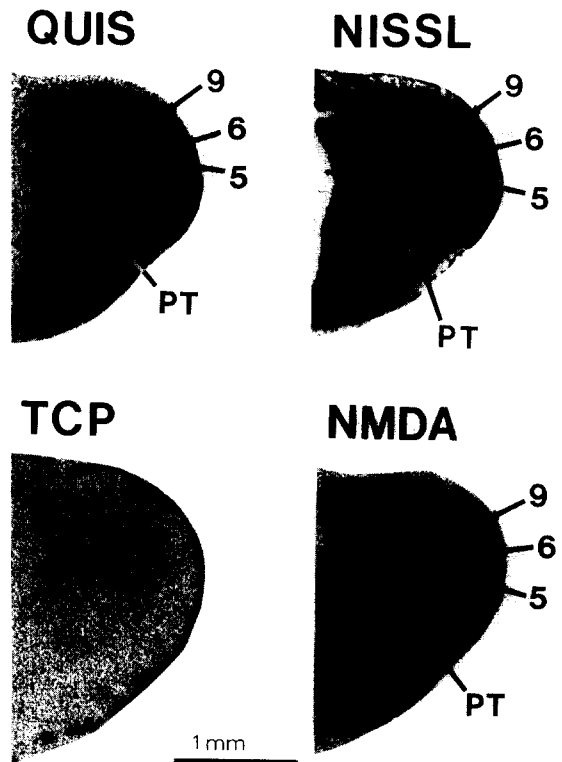


Fig. 1. Representative autoradiographic localization of [³H]TCP binding to PCP receptors and [³H]glutamate binding to quisqualate and NMDA receptors in the juvenile frog tectum. A tissue section stained for Nissl substance is included for comparison. For [³H]TCP binding¹², sections were preincubated for 30 min in ice-cold 50 mM Tris-acetate, pH 7.4 and then dried. Sections were then incubated for 45 min in the same ice-cold buffer with 1 mM magnesium acetate and 30 nM [³H]TCP in the presence or absence of 20 μM TCP. Magnesium (1 mM) was added to the incubation medium, as this concentration has been shown to maximally increase [³H]TCP binding^{2,12}. Rat brain sections were assayed in the same experiment to ensure that no problems had occurred in the [³H]TCP binding assay. Sections were then washed 3 times, 1 min each, in ice-cold buffer with Mg^{2+} and rapidly dried. No specific [³H]TCP binding was observed. The dense outer band of [³H]TCP binding is an artifact of the embedding matrix. [³H]Glutamate binding was carried out under two different conditions that select for NMDA and quisqualate receptors⁷. For NMDA receptor binding, sections were preincubated in ice-cold Tris-acetate, pH 7.4 and dried. Sections were then incubated in the same ice-cold buffer with 40 nM L-[³H]glutamate plus 2.5 μM quisqualate in the presence or absence of 100 μM glutamate. Sections were rapidly washed 3 times with 2 ml of ice-cold buffer, followed by one rinse with a mixture of ice-cold glutaraldehyde and acetone (1:19 v/v) and then rapidly dried. Quisqualate receptor binding was carried out similarly except the buffer was 50 mM Tris-HCl and the incubation buffer included 40 nM [³H]glutamate plus 2.5 mM CaCl_2 and 100 μM NMDA. Under these conditions, QA-sensitive [³H]glutamate binding likely represents binding to postsynaptic neuronal QA receptors, rather than binding to 2-amino-4-phosphonobutyric acid (APB) receptors or chloride-driven vesicular uptake¹.

TABLE I

Specific binding of [³H]glutamate to NMDA and quisqualate sites in juvenile frog brain

Values represent the mean \pm S.E.M. of 4 animals. Autoradiography and quantification of autoradiograms were carried out as described in Fig. 1. No specific [³H]TCP binding was present in any areas studied.

Tectal areas	Ligand bound (pmol/mg protein)	
	NMDA	Quisqualate
Layer 9	0.51 \pm 0.03	0.93 \pm 0.06
Layer 6	0.08 \pm 0.02	0.28 \pm 0.01
Layer 5	0.18 \pm 0.03	0.75 \pm 0.07
Pretectum	0.84 \pm 0.10	4.80 \pm 0.49

binding assays) and are expressed in pmol/mg protein (mean \pm S.E.M.). [³H]Glutamate (53 Ci/mmol) and [³H]TCP (47.6 Ci/mmol) were obtained from New England Nuclear.

NMDA-sensitive and QA-sensitive [³H]glutamate receptor-specific binding was heterogeneously distributed throughout the normal frog tectum (Fig. 1; Table I). QA-sensitive glutamate binding was 45–88% higher in all tectal regions studied in comparison to NMDA-sensitive glutamate binding. In contrast, autoradiographic analysis of [³H]TCP binding sites revealed no specific binding in any of the regions studied. NMDA and QA receptor binding was most concentrated in the pre-tectum (NMDA = 0.84 \pm 0.10 pmol/mg protein; QA = 4.80 \pm 0.49 pmol/mg protein). Within the tectum, layer 9, the retinotectal synaptic zone, had the highest density of binding (NMDA = 0.51 \pm 0.03 pmol/mg protein; QA = 0.93 \pm 0.06 pmol/mg protein). Cellular layer 6 had the least amount of binding (NMDA = 0.08 \pm 0.02 pmol/mg protein; QA = 0.28 \pm 0.01 pmol/mg protein). Intermediate binding was found in the deep fiber layer 5 (NMDA = 0.18 \pm 0.03 pmol/mg protein; QA = 0.75 \pm 0.07 pmol/mg protein). There were no differences in the amount of NMDA or QA receptor binding between normal and 3-eyed frogs (layer 9: NMDA = 0.51 \pm 0.03, normal vs 0.48 \pm 0.02, 3-eyed; QA = 0.93 \pm 0.06, normal vs 0.88 \pm 0.04, 3-eyed; independent two-tailed Student's *t*-test).

These results demonstrate that NMDA- and QA-sensitive glutamate binding is heterogeneously distributed within the frog tectum. The high density of NMDA and QA receptor binding in two RGC

termination sites, the retinotectal synaptic zone (layer 9) of the optic tectum and the pretectum, is consistent with evidence that this pathway is glutamatergic. These data are also consistent with studies indicating that chronic NMDA receptor activation and blockade affect the plasticity of this system. The similar localizations of NMDA- and QA-sensitive binding sites suggests that a cooperative interaction of the different glutamate receptor subtypes may occur, as seen in other systems. For instance, activation of non-NMDA glutamate receptors may provide sufficient membrane depolarization to permit ion conductance through the voltage-sensitive NMDA receptor/channel¹¹. The density of both NMDA and QA receptor binding in layer 9 is equivalent in normal and 3-eyed frogs. This may reflect a comparable number of synapses in the neuropil of normal and 3-eyed animals⁵.

No specific [³H]TCP binding was observed in layer 9 or in any brain region examined in the juvenile frog. Similarly, no TCP binding sites were seen in goldfish brain⁸. These findings are in contrast to the identical distribution of NMDA receptors and PCP receptors in adult rats¹². It may be that the PCP receptor is not expressed in early post-metamorphic frogs. Our preliminary studies in the developing rat suggest that expression of [³H]TCP binding lags behind the expression of NMDA receptor binding in rat forebrain (manuscript in preparation). Furthermore, specific [³H]TCP binding is present in adult frog brain homogenates and the non-competitive antagonist, ketamine, blocked NMDA-mediated electrophysiological responses in frog spinal cord^{13, 19}. In adult rat brain, low- and high-affinity [³H]TCP binding sites are observed. Spinal cord, brainstem and cerebellum have predominantly low-affinity binding sites, whereas high-affinity sites predominate in forebrain. Except in cerebellum, NMDA-receptors appear co-localized with high-affinity sites. It is possible that low-affinity sites, not measured in the autoradiographic assay, predominate in juvenile brain. The relationship of low affinity sites to the functional NMDA receptor is unclear. It is possible, therefore, that the non-competitive NMDA receptor antagonists acting at the PCP receptor, in contrast to competitive antagonists, will not alter the development of ocular dominance column formation in 3-eyed frogs.

We have previously shown that glutamate receptors, and the NMDA subtype in particular, are involved in the development of the retinotectal projection. Here we demonstrate a high density of glutamate receptor binding sites in a system widely recognized for its developmental plasticity. These data support our hypothesis that glutamate receptors, and the NMDA receptor/channel in particular, are involved in the fine tuning of the retinotectal

projection. The data also support the role of excitatory amino acids neurotransmitters in the retinotectal projection of the frog.

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